Maize U2 snRNAs: gene sequence and expression

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Received September 5, 1989; Revised and Accepted October 17, 1989

EMBL accession no. X16459

ABSTRACT

The complexity of plant U-type small nuclear ribonucleoprotein particles (UsnRNPs) may represent one level at which differences in splicing between animals and plants and between monocotyledonous and dicotyledonous plants could be effected. The maize (monocot.) U2snRNA multigene family consists of some 25 to 40 genes which from RNA blot and RNase protection analyses produce U2snRNAs varying in both size and sequence. The first 77 nucleotides of the maize U2-27 snRNA gene are identical to U2snRNA genes of *Arabidopsis* (dicot.). Despite much lower sequence homology in the remaining 120 nucleotides the secondary structure of the RNA is conserved. The difference in splicing between monocot. and dicot. plants cannot be explained on the basis of sequence differences between monocot. and dicot. U2snRNAs in the region which may interact with intron branch point sequences.

INTRODUCTION

Variation in the levels of transgene expression encountered over the last five years has led to an increased interest in fundamental aspects of plant gene expression. Transcription, pre-mRNA processing, mRNA stability, transport to the cytoplasm and translation are all levels at which gene expression may be regulated. In particular, pre-mRNA splicing has been implicated in the poor expression of some monocotyledonous (monocot.) genes when transformed into dicotyledonous (dicot.) plants (1). The importance of introns and splicing in gene expression is further highlighted by observations that certain plant introns may enhance expression levels (2,3) as has also been shown in some animal gene systems (4).

The interruption of protein coding genes by intron sequences is a feature of eukaryotic genomes. Removal of these intron sequences from messenger RNA precursors (pre-mRNAs) by the process of splicing is required for gene expression. Due to the availability of animal and yeast *in vitro* splicing systems (5–8) the mechanism of splicing and the various factors involved in the splicing process have been extensively studied (recently reviewed in Refs. 9 and 10). The initial event in splicing is the assembly of a large ribonucleoprotein complex on the pre-mRNA, known as the spliceosome. The assembly of the spliceosome requires both conserved sequence elements found near the splice junctions of introns and U-type small nuclear ribonucleoprotein particles (UsnRNPs) (9,10).

In the absence of a plant *in vitro* splicing system, pre-mRNA splicing in plants has been investigated at four different levels. Firstly, consensus sequences for the 5' and 3' splice junctions have been derived and, although generally similar to those of animals and yeast, the polypyrimidine stretch found upstream of the 3' splice site in most animal introns is much less apparent in plants (11–13). When compared, monocot. 3' splice sites tended

to contain more pyrimidine bases directly upstream to the -AG dinucleotide than dicot. introns (12). However, the polypyrimidine stretch required for splicing of animal introns (14) is not essential for plant intron splicing and its function may be replaced by the AU-rich feature of plant introns (15). Secondly, the splicing of a number of plant introns has been examined in the HeLa cell *in vitro* splicing system (13,16–18). Although the majority were successfully spliced, where splicing did not occur or was inefficient it appeared that the plant 3' splice site was poorly recognised by the animal splicing machinery (13). Thirdly, splicing in animals and yeast is mediated by UsnRNPs which consist of one or two UsnRNA moieties in association with a number of protein molecules (9,10,19). A number of plant UsnRNAs have been directly sequenced (20–25) and more recently plant UsnRNA genes have been cloned, sequenced and their expression analysed (26–29). Finally, the lack of a plant *in vitro* splicing system has been to some extent compensated for by the development of transient transformation methods where intron constructs are introduced into plant protoplasts and splicing analysed. The successful splicing of both synthetic and natural introns using this approach is providing detailed information on plant pre-mRNA splicing (13,15,18,30). Although similarities in intron consensus and UsnRNA sequences among plants, yeast and animals are apparent the lack of splicing of animal introns in plant tissue suggests basic differences in the mechanism of splicing between animals and plants (13,31).

Plant UsnRNAs display a level of sequence variation far greater than that observed for animal UsnRNAs (28). Their complexity has been clearly demonstrated in the sequences of pea U5snRNAs (20) and *Arabidopsis* U2snRNA gene variants (28), and by two-dimensional gel electrophoresis (32) and Northern analyses (33). The significance of this UsnRNA sequence variation, given the role of UsnRNPs in the recognition of the splice site and branch point sequences and spliceosome formation (9,10,19) is unknown. In light of the potential for base pairing interactions between the UsnRNA moieties of the U1 and U2 snRNPs and the 5' splice site and branch point sequences respectively (34–39) plant UsnRNA sequence variation is being investigated. As yet all available plant UsnRNA sequences are of dicotyledonous origin. In this paper we report the sequence of a maize U2snRNA gene and an analysis of maize U2snRNA gene expression. Although U2snRNAs have been partially sequenced from wheat (21) and other monocotyledons (28) this paper reports the first UsnRNA gene sequence from a monocotyledonous plant.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from Pharmacia, Koch-Light and Cambio. T4 DNA ligase, alkaline phosphatase, Klenow enzyme, RNase A and T₁, SP6 polymerase and RNase inhibitor were obtained from Boehringer-Mannheim. Zeta probe was from Bio-Rad and radionucleotides were from Amersham.

Isolation of maize U2snRNA gene

A Bam HI maize genomic library in lambda EMBL4 was screened using standard procedures with the 560 base pair (bp) Hind III fragment of the *Arabidopsis* U2 clone, pGEMU2.22 (28) (a kind gift from Drs. P. Vankan and W. Filipowicz, Friedrich Miescher Institute, Basel). A U2-containing lambda clone, MzU2-27, was isolated (Fig. 1a) and a 6.1 kilobase pair (kbp) Bam HI–Hind III fragment was subcloned into pUC18 to generate pMzU2-27.O8. A 1.0 kbp Xba I fragment containing the maize U2snRNA gene was further subcloned into pUC18 to produce pMzU2-27.O12. These plasmids, pMzU2-27.O8 and

Fig 1. Cloning of the maize U2-27 gene. a) Restriction map of the Lambda EMBL4 clone, lambda MzU2-27. b) and c) Restriction maps of subclones of lambda MzU2-27 – plasmids pMzU2-27.08 and pMzU2-27.012. The position and direction of transcription of the U2 gene are indicated. The bar in c) identifies the 560 bp Xba I–Xho I fragment used in *in vitro* transcription constructs. B–Bam HI, C–Cla I, E–Eco RI, H–Hind III, Hc–Hinc II, R–Eco RV, X–Xba I and Xh–XhoI.

pMzU2-27.012, shown in Fig. 1b and c, were used to produce the complete sequence of the coding region and 279 bp and 218 bp of 5' and 3' flanking regions respectively. Sequencing was carried out by the chain termination method with Sequenase using primers complementary to positions 4 to 21 and 68 to 51 or by the base-specific chemical cleavage method (40).

Subclones for *in vitro* transcription were constructed by cloning the 560 bp Xba I–Xho I fragment (Fig. 1c) into the Xba I and Sal I sites of pSP64 and pSP65 (41) to generate pMzU2-27.022 and pMzU2-27.023 respectively.

Preparation of RNA transcripts

The SP6 plasmids, pMzU2-27.022 and pMzU2-27.023, were linearised at the Eco RI and Hind III sites respectively. *In vitro* transcription was carried out with 2 µg of linearised plasmid in a 40 µl reaction volume containing 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 100 µg/ml BSA, 500 µM each of ATP, CTP and GTP and 20 µM UTP, 20 µCi [³²P] UTP, 10 units SP6 polymerase and 50 units of RNase inhibitor at 40°C for 75 mins. (41). RNA transcripts were gel purified (14) prior to RNase A/T₁ protection analyses.

RNA extraction and RNase A/T₁ protection assays

RNA was prepared from endosperm of the maize line A619 using hot guanidinium isothiocyanate/phenol (42) and from cotyledon and root material of the maize line B73 as described (43). 20 µg of total RNA was precipitated with 100,000 dpm of [³²P]-labelled RNA transcripts representing the coding or complementary sense of the maize U2-27 snRNA gene. The RNAs were dissolved in 10 µl of 80% formamide, 400 mM NaCl and 10 mM Pipes, pH 6.4, denatured at 90°C for 2 min. and hybridised at 45°C for 16 hours. The RNase A and T₁ digestion was carried out by the addition of 100 µl of RNase buffer (10 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 5 mM EDTA) containing 8 µg/ml of RNase A and

0.4 µg/ml of RNase T₁ at 26°C for 40 min. (30). The reaction was stopped by the addition of 2 µl 10% SDS and 3 µl Proteinase K (10 mg/ml) and incubated at 37°C for 15 min.. Following phenol/chloroform and chloroform/isoamyl alcohol extractions the RNA duplexes were precipitated after the addition of 10 µg carrier tRNA. The RNA was separated on a 1m, 6% polyacrylamide-urea gel alongside a DNA sequence marker and autoradiographed.

Maize UsnRNA analysis

7 µg of total maize endosperm RNA was separated on an 8% polyacrylamide-urea gel. The RNA was electroblotted onto Zetaprobe for 30 min. at 80V, 0.6–0.8 A as described in the Bio-Rad Transblot manual. The blots were pre-hybridised in 6×SSC, 5 × Denhardt's, 0.5% SDS and 10mM EDTA for 2 h at 65°C and hybridised in 6×SSC, 5 × Denhardt's, 0.2% SDS, 10 mM EDTA and 10% Dextran sulphate containing a [³²P]-labelled U2snRNA probe isolated from a potato U2-containing plasmid, pPotU2-22.O2 (R. Waugh and J.W.S. Brown, unpublished data) for 16 h at 65°C. Blots were autoradiographed at –70°C for 72 h.

RESULTS

Isolation of maize U2snRNA gene

A lambda EMBL 4 clone containing a maize U2snRNA gene was isolated using the *Arabidopsis* U2 gene probe. A partial restriction map of this clone, Lambda MzU2-27, the position of the U2 gene and the direction of transcription is shown in Fig. 1a. The plasmids, pMzU2-27.08 and pMzU2-27.O12 used in sequencing are shown in Figs. 1b and c. The 560 bp Xba I–Xho I fragment used to construct pMzU2-27.O22 and pMzU2-27.O23 for *in vitro* transcription is shown in Fig. 1c.

Sequence of the maize U2snRNA gene

The coding and the 5' and 3' non-coding regions of the maize U2snRNA gene contained on the pMzU2-27 plasmids is given in Fig. 2a. The 5' and 3' ends of the coding region have been determined from the size of the RNase A/T₁ protected fragments (see below) and by comparison of the maize U2snRNA sequence to those of *Arabidopsis* (28) and is based on the high degree of sequence conservation in U2snRNA genes among different species (44). The maize U2-27 gene codes for an RNA molecule of 197 nucleotides.

The coding region is identical to the published *Arabidopsis* U2snRNA genes (28) over the first 77 nucleotides. In the next 120 nt the maize U2 sequence differs by 4 base insertions, 2 base deletions and 34 base substitutions such that homology with the *Arabidopsis* U2.3 sequence falls to 66% over this region. Comparison with the broad bean U2snRNA sequence (24) showed virtual identity over the first 75 nt with the exception of the deletion of a U at position 7 and the substitution of a U for a C at position 51 in the broad bean sequence. From position 76 to the end of the molecule the maize sequence differs by 5 base insertions, 3 base deletions and 17 base substitutions giving a homology over this region of 79%. Similarly, the maize sequence differs from the partial (96 nt from the 3' end) pea U2snRNA sequence (20) by 3 insertions, 1 deletion and 19 substitutions giving a homology in the 3' end of 76%. The sequence differences do not alter the ability of the maize U2snRNA to form the secondary structure (Fig. 2b) proposed for the human U2snRNA (45). Analysis of the secondary structure of the maize U2-27 snRNA with the University of Wisconsin FOLD programme produced the above structure with a ΔG value of –55 kcal mol^{–1}.

Conserved sequences for plant UsnRNA genes have been identified in both the 5' and

3' non-coding regions from the sequences of *Arabidopsis* U2 and U5, bean U1 and soybean U1 snRNA genes (29). A comparison of this plant UsnRNA consensus and the *Arabidopsis* U2snRNA consensus with the corresponding sequences in the maize U2snRNA gene is shown in Fig. 2c. The sequence element at positions -66 to -76, corresponding to the -68 to -78 element which is required in *Arabidopsis* for enhanced levels of expression, differs by a single nucleotide, C instead of A at position -69. The basic TATA sequence is intact (positions -30 to -33) but there is variation in the surrounding sequences. The maize cap site adjacent sequence (positions -1 to -6) fits the *Arabidopsis* U2 consensus perfectly while the 3' end adjacent sequence differs from the other consensus sequences at a single nucleotide (Fig. 2c).

Expression of maize UsnRNAs

The number of maize U2snRNA genes or hybridising sequences has been estimated from Southern analyses of maize genomic DNA at approximately 25 to 40 (R. Waugh and J.W.S. Brown, unpublished data). Expression of U2snRNAs in maize has been examined by RNase A/T₁ protection and Northern blot analyses. The results of RNase A/T₁ protection analysis using total maize endosperm, cotyledon and root RNA and [³²P]-labelled RNA transcripts representing the coding or complementary sense of the maize U2-27 gene are shown in Fig. 3. Six groups of bands (Fig. 3, A-F) representing protected fragments were observed. A full length protected RNA fragment of 197 nt is seen in the group of bands in the size range 194–197 nt (Fig. 3,A) with the RNA probe complementary to the maize U2-27 sequence (Fig. 3, lanes 1–3). The other groups of protected fragments are approximately 148 to 151 nt (B), 135 to 139 nt (C), 121 to 126 nt (D), 98 to 101 nt (E) and 50 to 51 nt (F) in length (Fig. 3, lanes 1–3). The sizes of the protected fragments cannot be exactly determined from the DNA sequence marker as RNA fragments run slightly slower than DNA fragments in polyacrylamide-urea gels. No protected RNA fragments were obtained with the [³²P]-labelled RNA probe corresponding to the coding sequence of the maize U2-27 gene (Fig. 3, lanes 4–6) or when maize RNA was substituted with *E. coli* tRNA (results not shown).

The results of a high resolution Northern analysis of total maize endosperm RNA is shown in Fig. 4. Three major and one minor U2snRNA bands of approximately 195 to 202 nt in length were observed (Fig. 4, lanes 1 and 2). This size range will again be slightly overestimated as discussed above.

DISCUSSION

Structure of the maize U2snRNA gene

The sequence of the maize U2-27 snRNA gene is virtually identical to the *Arabidopsis* and broad bean U2snRNA sequences over the first seventy five nucleotides. Homology with *Arabidopsis* U2snRNA gene sequences and the broad bean and partial pea U2snRNA sequences falls over the remainder of the molecule to between 66% and 79%. The most highly conserved regions among all eukaryotic U2snRNA sequences available (44) are the first 70 nucleotides, the Sm antigen binding site, and the third and fourth single stranded

Fig 3. RNase A/T₁ protection analysis. RNA fragments protected from RNase A/T₁ digestion following hybridisation of total maize endosperm, cotyledon and root RNA with (³²p)-labelled maize U2-27 RNA probes are shown for the U2 complementary probe (lanes 1–3 respectively) and the coding sense probe (lanes 4–6 respectively). The DNA sequence marker (lanes A, C, G and T) is the sequence of the maize U2-27 gene using the oligonucleotide primer complementary to positions 68 to 51. The DNA sequence (lanes a, c, g and t) was loaded slightly later than the other lanes and is included to show that the faint bands in lanes 5 and 6 are due to overflow from the A sequencing reaction (lane a).

Fig 4. Maize RNA blot analysis. Total maize endosperm RNA was separated on an 8% polyacrylamide gel, alongside a DNA sequence marker, electroblotted and hybridised with a potato U2snRNA gene probe (lanes 1 and 2—lane 2 is a shorter exposure and enlargement of lane 1). The DNA sequence marker (lanes A and C) are the A and C reactions of the sequence of the maize U2-27 gene using the oligonucleotide sequence primer complementary to positions 68 to 51. The three major and one minor hybridising bands are arrowed.

loop sequences. These regions are all conserved in the maize U2snRNA sequence presented here with the exception of the Sm antigen binding site, AUUUUUUAG (46) which is AUUUUUUGU in Mz U2-27. The majority of the sequence differences between the maize and *Arabidopsis*, broad bean and pea U2snRNA sequences are in the area of the stems of the third and fourth stem-loops in the secondary structure (Fig. 2b). This is also the region where the majority of the sequence variation between the six *Arabidopsis* U2snRNA gene variants are apparent (28). The maize U2-27 snRNA would have the 3' end sequence of -CCAAC compared to -CCCGC found in 50% of the *Arabidopsis* sequences (28), -CCCAC in the broad bean sequence (24) and -CCCAA in the pea sequence (20). Despite these sequence differences the maize U2snRNA can still form the secondary structure proposed for the *Drosophila* U2snRNA (45). The primary sequence homology in the 5' half of the molecule suggests that the dual function of the U2snRNA in spliceosome formation (39,47) could also apply in plant pre-mRNA splicing. The conservation of secondary structure also points to conservation of function in plants. However, the actual

role of U2snRNPs in plants is still to be determined in light of the finding that synthetic introns lacking a branch point sequence are spliced following transient transformation into plant protoplasts (15).

UsnRNA genes require UsnRNA promoter and terminator sequences for transcription (48,49). Conserved promoter elements upstream of the coding region and a conserved sequence immediately following the 3' end of the coding region have been defined for *Arabidopsis* U2snRNA genes (28) and for plant UsnRNA genes as a whole (29) (Fig. 2c). The corresponding sequences in the maize U2snRNA gene fit these consensus sequences well showing single base differences in the -68 to -78 (28) element and in the 3' conserved sequences (Fig. 2a and c). The -68 to -78 sequence is 100% conserved in all six *Arabidopsis* U2snRNA gene sequences (28) and therefore the differences in the maize U2-27 snRNA may reflect either basic differences in maize UsnRNA genes or sequence variation within the maize U2snRNA gene families.

Variation in maize U2snRNAs

The complexity of plant UsnRNAs has been demonstrated by electrophoretic methods (32,33) and further shown by the sequencing of UsnRNAs themselves (20) or their gene families (28). As pointed out by the latter authors, the sequence variation observed in the *Arabidopsis* U2 multigene family is the greatest so far observed in any eukaryotic UsnRNA family (28). The RNA blot analysis presented here has shown three major and one minor U2snRNA hybridising bands in the range of 195–202 nt. Although this size range is a slight overestimate it agrees with the sizes of the maize U2-27 snRNA (197 nt), the *Arabidopsis* U2snRNA genes (195/196 nt) (28) and the broad bean U2snRNA (194 nt) (24). A previous RNA blot analysis which suggested that U2snRNAs in maize were larger than 208 nt (33) is also likely to be overestimated. The differences in intensity of the hybridising bands are probably due to the abundance of U2snRNAs of each size class and may reflect either different numbers of genes coding for the different size variants or differential expression of certain variants. The RNA blot analysis only provides information on the size of the U2snRNAs expressed in maize. The RNase A/T₁ analysis, on the other hand, provides some information on the sequence variation of U2snRNAs expressed in maize. In the first place the presence of a full length protected fragment suggests that the maize U2-27 snRNA gene or another gene of virtually identical sequence is expressed in maize endosperm, coleoptiles and root (Fig. 3). The groups of protected bands (Fig. 3, B–E) reflect variants of the maize U2snRNA family which differ from the maize U2-27 snRNA sequence. It cannot be determined whether each individual band represents a sequence variant or whether the scatter of bands within each group may be due to variations in digestion of the RNA hybrids by the RNases. The latter effect may explain the intensity of the group of bands of 99 to 102 nt (Fig. 3, E). These bands could correspond in size to digestion at the Sm antigen binding site which, although a highly conserved region, reflects an area of high AU content allowing greater accessibility to the RNA hybrids by the RNases. The differing intensities of the various signals may be due to different numbers of genes containing a particular sequence variation or different levels of expression of individual sequence variants. The bands of 50 and 51 nt (Fig. 3, F) are probably internal protected fragments. The groups of bands B, C and D show that the majority of sequence variations in the maize U2snRNA family occur between positions 120 and 152 as has been noted for the *Arabidopsis* U2snRNA gene family (28) and the potato U2snRNA gene family (R. Waugh and J. W. S. Brown, unpublished data). Taken together, the results of the RNA blot and RNase A/T₁ analyses show that the maize U2snRNA gene family codes

for an array of both size and sequence variants as has been shown for the pea U5snRNAs (20) and *Arabidopsis* U2snRNA genes (28) and again points to the complexity of UsnRNA multigene families in plants.

The differences in splicing between animal and plant introns and between monocot. and dicot. introns may be reflected in such UsnRNA sequence variation and complexity. All of the plant UsnRNAs and their genes which have been sequenced to date are of dicot. origin (20–29). In light of the possible difference in splicing between monocots. and dicots. and the potential for base pairing between the 5' splice site and the 5' end of U1snRNAs (34–37) and the branch point sequence and internal nucleotides of U2snRNAs (38,39,47), the sequences of monocot. UsnRNAs have been awaited. The sequence of the maize U2-27 snRNA gene is identical to the *Arabidopsis* and broad bean U2snRNAs over the first 75 nt which contains the region of the U2snRNA (positions 32–37) which may interact with the branch point sequence (38,39). On the basis of this sequence similarity and that of the sequence of the 5' region of wheat U2snRNAs (28) the reported splicing differences are unlikely to be due to UsnRNA sequence variation at least for the branch point/U2snRNA interaction.

ACKNOWLEDGEMENTS

We would like to thank Dr. W. Powell for his support. We are grateful to Mrs. G. Clark for her excellent technical assistance and to Mr. C. Simpson for the FOLD analysis of the maize U2snRNA. We are indebted to Drs. P. Vankan and W. Filipowicz for the kind gifts of the *Arabidopsis* U2.3 gene and U2 sequencing primers. This research was supported by grants to Dr. J. W. S. Brown from the Agricultural and Food Research Council and the Nuffield Foundation.

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